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EXPERIMENTAL BACTERIAL AND VIRAL DIARRHEA (EXPERIMENTAL VIRAL D--ETC(U)

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EXPERIMENTAL VIRAL DIARRHEA IN PIGS (U)

FINAL REPORT  
by

Luis R. Otero-Vilardebó, Ph.D.  
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) <p>This investigation was undertaken to develop a system which would enable us to induce viral and/or bacterial diarrhea in pigs at will, and in turn, to study the physiological and morphological changes induced by the pathogen preceding the overt signs of diarrhea.</p> <p>A strain of transmissible gastroenteritis virus (TGE) obtained from M. Pensaert at Purdue University induces diarrhea in young (5 to</p>		

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15 day old) pigs within 48 hrs. after oral administration.

The virus is maintained by serial passage in young pigs. The intestinal mucosa and contents are homogenized and TGE virus harvested by centrifugation. 0.5 ml of the centrifuged homogenates suffice to infect young pigs with 100 percent efficiency. Thus far, only orally administered virus have produced diarrhea. Identical doses administered intraperitoneally were ineffective.

Diarrhea is usually accompanied by vomiting. No bloody stools have been observed. Examination of the intestinal mucosa with a dissection microscope did not reveal any shedding of the mucosa. Histological examination at 48 hrs, when diarrhea was already overt, revealed that the mucosal continuity was undisturbed. However, the cytoplasm of the mucosal cells is less basophilic and has a spongy appearance.

To study the earliest stages of infection, TGE virus was introduced directly into an intestinal loop in situ, and allowed to remain there from 2 to 24 hrs. before the animals were sacrificed. Histological studies of samples taken from a loop 2 hrs. after infection revealed a dilatation of the Golgi apparatus and the endoplasmic reticulum of the apical cytoplasm. The relation of this early change to the process of viral invasion remains to be elucidated.

Disaccharidase activity in homogenates of intestinal mucosa from control and infected pigs has been studied by Dahlquist's method, and using lactose, maltose sucrose, thehalose, isomaltose, and cellubiose as substrates.

All enzymes were greatly reduced at 24 and 48 hrs. after oral infection. Lactase activity was reduced to less than 25 percent of the control levels.

Jejunum was the region of the digestive tract where the reduction in enzyme levels was greatest.

Colonic mucosa exhibited very low enzymatic activity in control animals.

The results of Lactose Tolerance Tests performed on control and TGE infected pigs suggest that Lactose absorption is markedly impaired 48 hrs. after oral infection.

## SUMMARY

This investigation was undertaken to develop a system which would enable us to induce viral and/or bacterial diarrhea in pigs at will, and in turn, to study the physiological and morphological changes induced by the pathogen preceding the overt signs of diarrhea.

A strain of transmissible gastroenteritis virus (TGE) obtained from M. Pensaert at Purdue University induces diarrhea in young (5 to 15 day old) pigs within 48 hrs. after oral administration.

The virus is maintained by serial passage in young pigs. The intestinal mucosa and contents are homogenized and TGE virus harvested by centrifugation. 0.5 ml of the centrifuged homogenates suffice to infect young pigs with 100 percent efficiency. Thus far, only orally administered virus have produced diarrhea. Identical doses administered intraperitoneally were ineffective.

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## FOREWORD

In conducting the research described in this report, the investigator adhered to the "Guide for the Laboratory Animals Facilities and Care", as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences-National Research Council.

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## INTRODUCTION

Enteric diseases continue to be one of the three major causes for admissions among military personnel throughout the world. Among the active duty military personnel of the U.S. Air Force, a total of 18,884 cases of enteric diseases were reported from July 1967 to June 1969. Only 965 (5.2 percent) of all the cases were attributable to specific protozoan or bacterial agents. The diagnostic procedures do not include tests for possible viral agents, and therefore there is no data available on the frequency with which viruses are the causative agents. A total of 17,919 cases (94.8 percent) were grouped as Unspecific Gastroenteritis and Colitis. It is painfully obvious that we are still very far from complete diagnosis of the enteric diseases, and that only a very small proportion of the patients can be treated with full understanding of their ailment.

The development of effective methods for the treatment of viral diarrhea will be greatly accelerated by an understanding of the mechanisms of viral invasion of the intestinal mucosa and of the physiological and morphological changes induced by the virus.

This may be best achieved by studying a system in which diarrhea can be induced at will. The Transmissible Gastroenteritis (TGE) in pigs is such a system. Diarrhea develops within 48 hrs. in all young pigs on oral dose of the TGE virus.

Pig is the animal of choice for this type of study because, like man, it is omnivorous.

The objectives of the present study are to record the physiological and morphological changes induced by the TGE virus in the digestive tract of young pigs, prior to the overt signs of diarrhea.

## MATERIALS AND METHODS

Animals: Young pigs, (less than 30 days old) were obtained from local farms, and kept in the laboratory for 24 hrs. before they were used. They were kept in individual stainless steel cages with a false wire-mesh floor. Droppings fell through the mesh to a tray partially filled with a surgical dessinfectant, Wescodyne G. The animals were fed on a milk-formula diet containing evaporated milk, dextromaltose and water.

Virus Stock: A culture of Transmissible gastroenteritis (TGE) virus obtained from Purdue University has been kept and multiplied in our laboratory by serial passages in young pigs. Diarrhea was always evident within 48 hrs. after the pigs received an oral infective dose of TGE virus. Animals were then killed by an overdose of Nembutal. The entire intestine was removed and kept frozen at  $-65^{\circ}\text{C}$  for not less than a week. The material was then thawed at  $4^{\circ}\text{C}$ , and the mucosa and the intestinal contents were suspended in 100ml of 1.0M phosphate-buffered saline containing 1,000 units of Penicillin and 2.5mg of Streptomycin per milliliter. The suspension was homogenized with a motor driven glass homogenizer at  $4^{\circ}\text{C}$ , and centrifuged at 12,000 g for 15 min. at  $4^{\circ}\text{C}$ . The supernatant was resuspended and recentrifuged twice at 32,000 rpm for 90 mins. in a fixed-angle type rotor #40 and a Spinco Model L centrifuge. The supernatant was discarded, and the pellet was resuspended in 20 ml of 0.1 M Phosphate Buffered Saline (PBS). This suspension of partially purified virus constituted our stock solution which was used for reinfections.

Purification of the TGE virus: The stock solution was further purified by centrifugation in a discontinuous sucrose gradient. This gradient was prepared by carefully layering 1ml layers of 0.1 M phosphate buffer containing 500, 400, 300, and 200 mg of sucrose respectively. A 1 ml sample of the virus stock was layered at the top of the gradient and centrifuged at 32,000 rpm for 65 minutes, using a preparative centrifuge with a swing-bucket rotor (SW-39). The centrifuge was slowly accelerated up to 10,000 rpm to minimize the mixing of the layers. The virus formed a distinct band at the interphase between the layers containing 200 and 300 mg of sucrose. This band was aspirated with a sterile hypodermic syringe in a 1.0 ml volume, and resuspended in 5ml of 0.1 M PBS. It was then centrifuged in a fixed angle rotor, type 40, at 32,000 rpm for 90 minutes. The sediment was resuspended in 2.0 ml of PBS, and kept frozen for further biochemical and morphological studies.

Ligated Intestine Preparations: Pigs were fasted for 18 hrs. before surgery. All surgical procedures were conducted under anesthesia induced by an intraperitoneal injection of 15 mg sodium pentobarbital per kilogram body weight. When necessary, anesthesia was maintained with ether by inhalation. Laparotomy was performed by a longitudinal lateral to the left nipples, and extending between the third and the fifth nipples. The ileum was located, divide into segments by means of string ligatures, and 1.5ml of TGE stock was injected into each segment. The intestines were



returned to the peritoneal cavity and the abdominal wall was closed by means of metal clips. Animals were maintained under sedation for the duration of the experiment, ranging from 2 to 24 hrs. During this time they were allowed only water. Respiratory difficulties occasionally caused death of very young pigs during the initial stages of anesthesia, but all other pigs recovered fully from the laparotomy.

Disaccharidase Activity: Control and infected pigs were fasted for 18 hrs. before surgery. Infected pigs were sacrificed at 24 and 48 hrs. after receiving an oral infective dose of TGE virus. Intestinal segments, approximately one inch long were resected, opened lengthwise, and samples of uniform size were obtained by punching the entire wall with a well-sharpened cork borer. The mucosa of the sample was then scraped off with a glass slide, and transferred to 10 ml of Kreb's Ringer solution. The mucosal scrapings were then homogenized in a Sorvall Omnimixer and centrifuged in arefrigerated centrifuge for 10 mins. at 6,000 g to eliminate cell debris. The supernatant was assayed for protein concentration using the Lowry method and for disaccharidase activity by the method of Dahlqvist. Maltose, lactose, sucrose, isomaltose, trehalose, and cellubiose were used as substrates. The specific disaccharidase activity was determined and expressed as moles of glucose liberated in one minute per gram of protein.

Microscopy: The intestinal mucosae of the experimental animals were examined under a dissecting microscope to determine any gross damage to the mucosa.

Samples of jejunum, ileum, and colon were taken from each of the infected animals, fixed in glutaraldehyde, and osmium tetroxide and embedded in Epon 812. Sections for light microscopy are cut at 1  $\mu$  and stained with methylene blue - Azure A.

Lactose Tolerance Test: Control and infected Pigs were fasted overnight, with water allowed ad libitum. Animals were sedated with 15 mg Nembutal per kg. body weight. The left femoral vein was uncovered and cannulated. A fine polyethylene tubing was passed into the stomach, and through it, the pigs were administered 1.75 gms. of lactose/kg of body weight. Blood samples (1.0 ml) were taken through the canula at selected intervals after intubation. Blood glucose was determined by the glucose-oxidase method. Infected pigs had received an oral dose of T.G.E. stock 24 or 48 hrs. prior to the lactose tolerance test.

## RESULTS AND DISCUSSION

Clinical Observations: Oral administration of 0.5 ml of the TGE stock produces overt signs of diarrhea within 48 hrs. The stock has proved virulent in 100 percent of the infections. Diarrhea is usually accompanied by vomiting. Stools are watery, yellowish, and bloodless. Animals lose their appetite, and have lost as much as 1 Kg body weight within 48 hrs.

Microscopical findings: Examination of the intestinal mucosa with a dissection microscope does not reveal any abnormal shedding of the mucosa, nor hemorrhagic lesions. Our histological findings indicate that the cell continuity remains undisturbed. These observations are compatible with the bloodless nature of the stools.

The mucosa of ileal loops infected in situ, revealed some alterations occurring within 2 hrs. after infection. The most striking of these is the dilatation of the apical endoplasmic reticulum and the Golgi Apparatus. The architecture of the villi is apparently unchanged at this stage.

Mucosal samples taken 48 hrs. after infection, revealed that the basophilia of the cytoplasm is altered. Cells at the base of the villi have a very strong basophilia, but the cells along the sides and apex of the villi have considerably less basophilia. These cells often exhibit a very spongy appearance.

These tissues are now being examined with the electron microscope to establish how the virus is associated with these changes.

Ligated intestinal loops: The instillation of viral homogenates into ligated intestinal loops in situ had two purposes: (1) to determine the course of viral invasion and replication in the mucosa, and (2) to determine whether the virus can produce a dilatation of ligated intestinal loops, similar to that produced by enteropathogenic *E. coli*. Thus far, we have not observed any dilatation of ligated ileal segments into which we introduced 1.5 ml of our TGE virus stock. In all experiments, the virulence of the TGE stock was assayed by oral administration of 0.5 ml to a control animal. The stock has proved virulent in 100 percent of the cases, producing overt diarrhea within 48 hrs. At this writing, the longest time that we have kept a ligated intestinal loop is 20 hrs. The loop remained undilated, and no water accumulated in the segments above or below the ligated loop.

Disaccharidase Activity: Marked changes in the enzyme contents of the intestinal mucosa of pigs were observed in animals which had received an oral infective dose of TGE virus.

In duodenum, all the sugars tested were less readily hydrolyzed by homogenates from infected pigs than from the controls (Fig. 1). Lactase activity was sharply reduced at 24 hrs. Maltase was moderately reduced at 24 hrs., but the effect was very pronounced at 48 hrs.

In jejunum, the levels in the controls are higher than the control values in duodenum (Fig. 2). Here, all the enzymes were greatly reduced in infected pigs at 24 and 48 hrs. Lactase activity was reduced to 2 percent of the control values.

The enzyme levels of the ileum of control animals were generally lower than those in the jejunum of the same animals. Maltase was the

enzyme most affected (Fig. 3).

The colonic mucosa of control animals exhibited very low enzymatic activity (Fig. 4). The level of lactase was higher in the animal 24 hrs. post infection than in the control. This may be due to bacterial contamination.

The enzyme most affected by viral infection was lactase (Fig. 5). The reduction was greatest in jejunum. The controls obtained from the four regions of the digestive tract are in very close agreement with the values reported by Dahlqvist (1961).

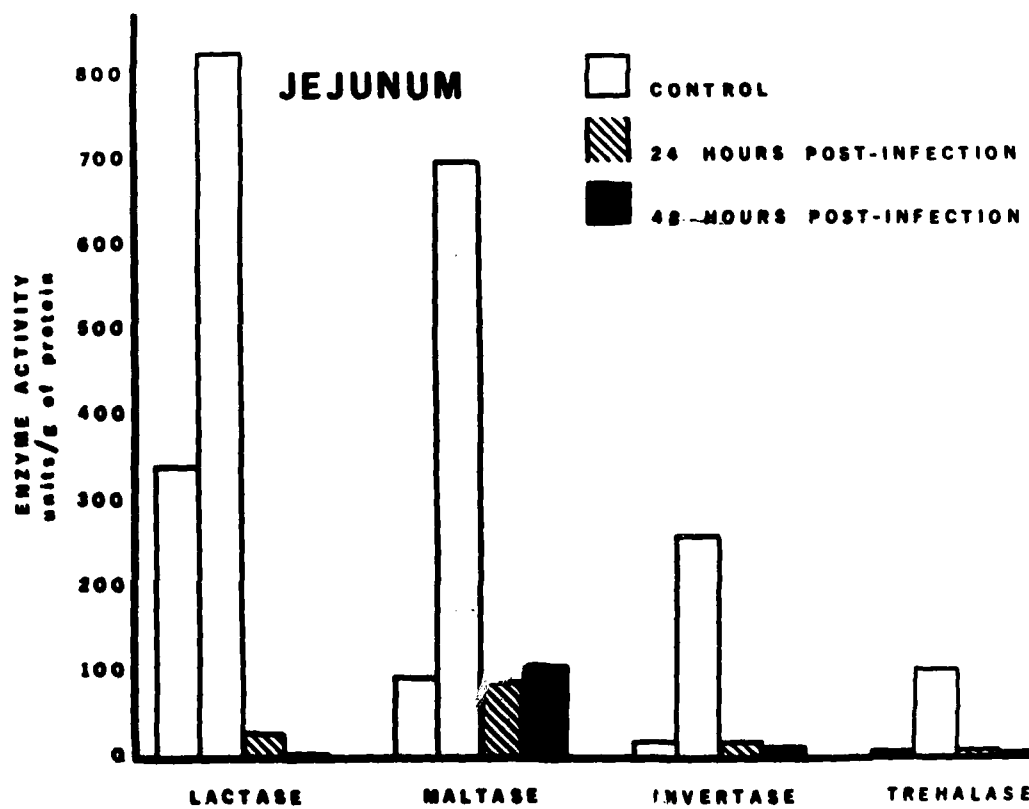
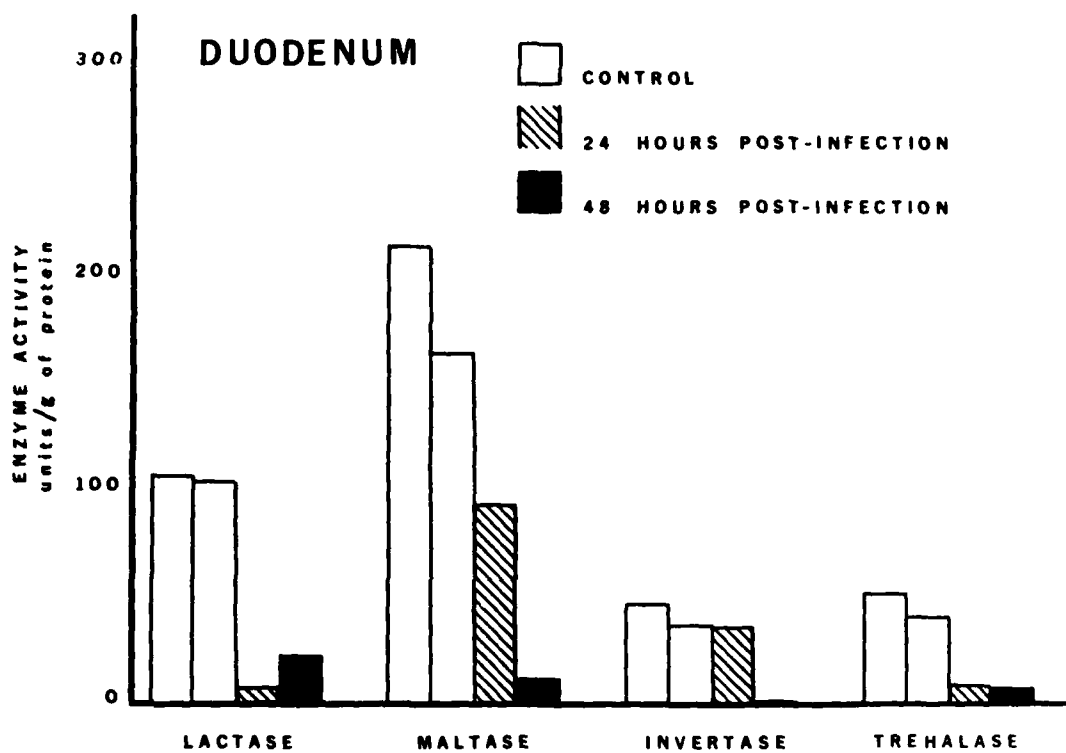
The results obtained in three experiments are summarized in Table I. In each experiment, the lactase activity of jejunum was reduced to less than 25 percent of the control values. The lactase activity in ileum was also reduced by more than 30 percent.

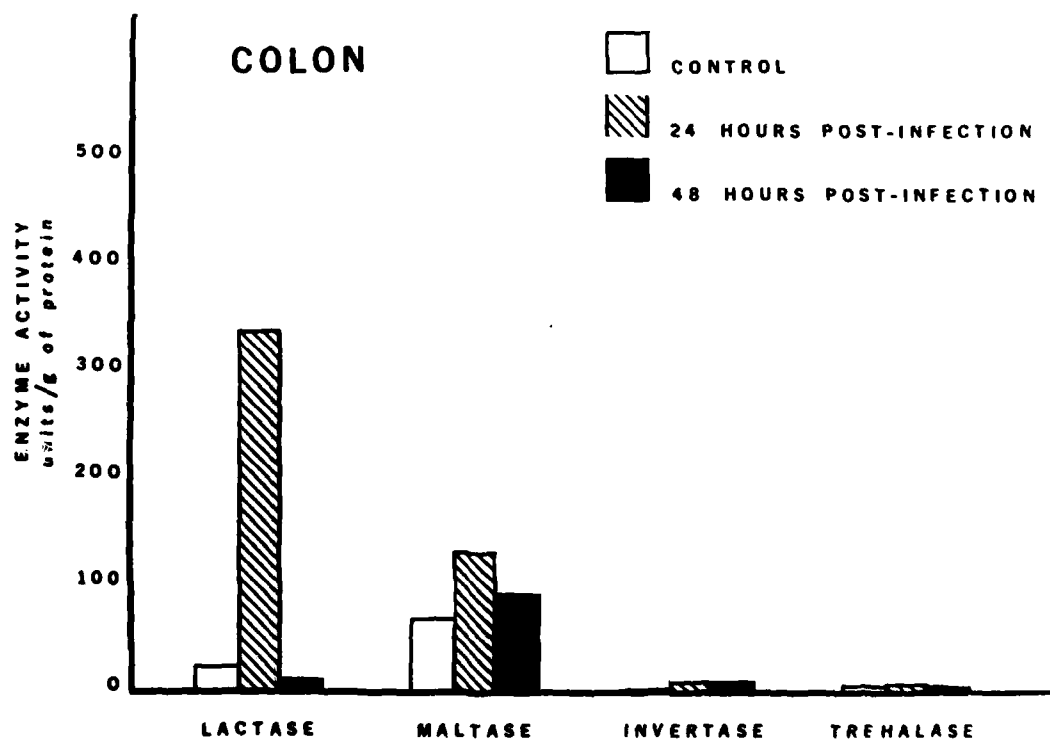
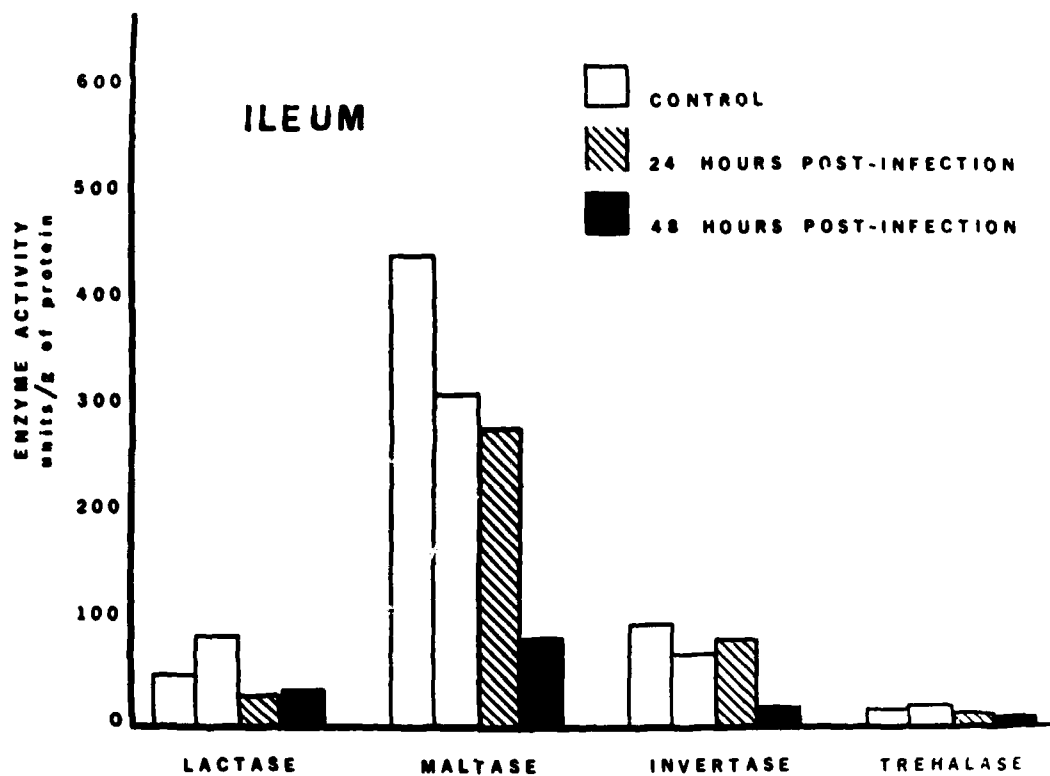
Maltase was altered to a lesser extent. In jejunum, the values were nearly normal in two of the experiments.

Invertase and Trehalase considerably reduced in all three experiments.

Lactose Tolerance Test: Blood glucose levels in Control Pigs underwent changes very much like those observed in a Glucose Tolerance test in man, with a sharp increase during the second thirty minutes followed by a sharp return to the normal values during the second hour (Fig. 6). In animals infected 48 hrs. prior to the Lactose Tolerance Test, the rise in blood glucose is totally absent, and actually a slight decline in blood glucose was observed.

The rise in blood sugar seen in man and control animals is interpreted as the result of sugar being absorbed at a rate faster than the liver's capacity to control blood sugar levels. The absence of the peak may be interpreted as an indication of impaired sugar absorption in the intestine. A reduced lactose absorption in the intestine of TGE infected animals consistent with the marked lactase reduction recorded before.





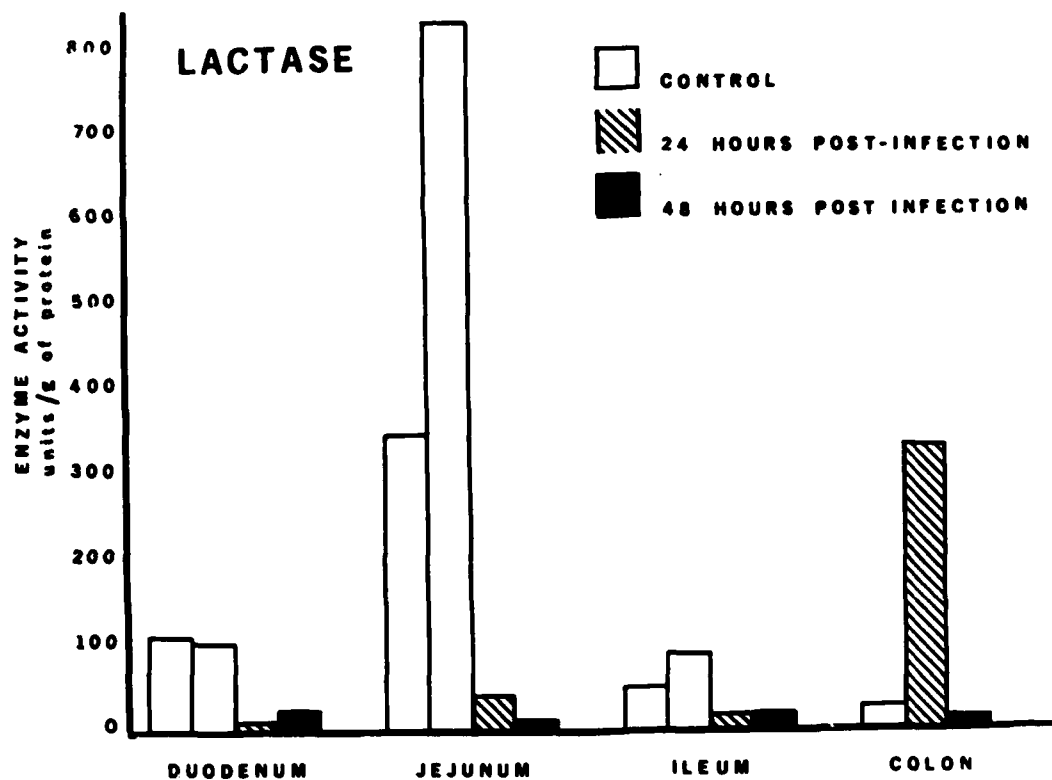
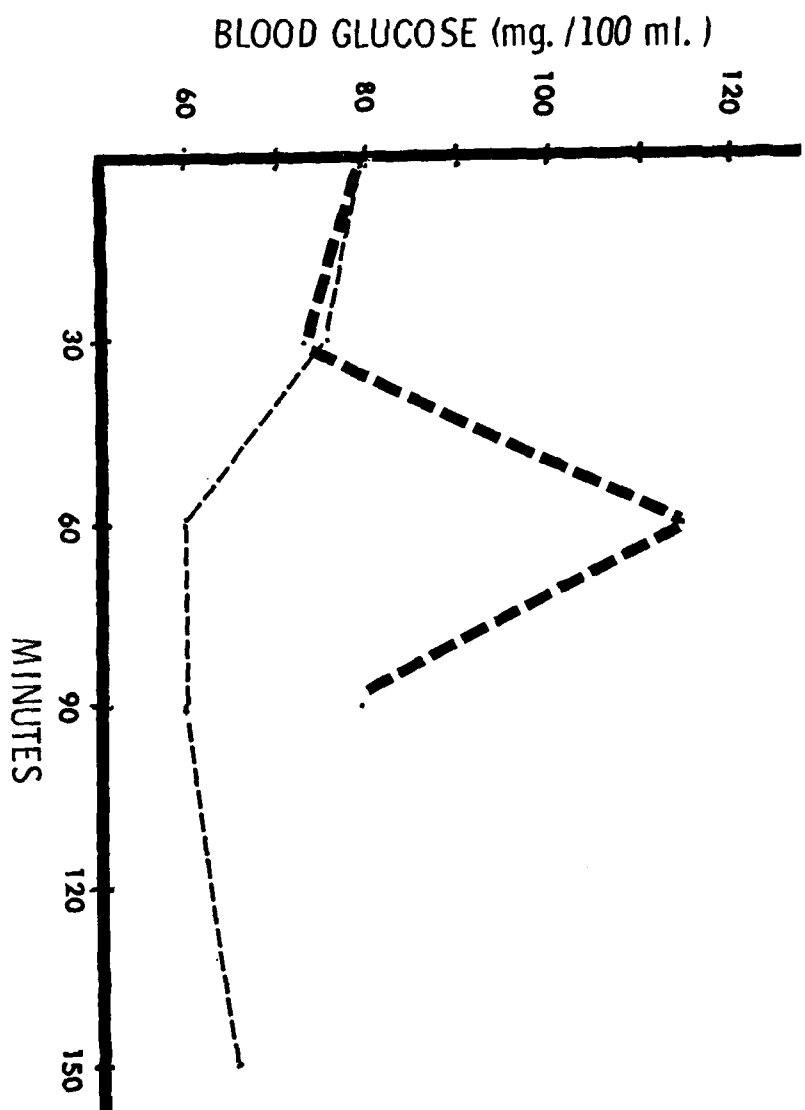


TABLE I

Date comparing enzyme levels in mucosal homogenates from TGE-infected pigs.  
 Values are expressed as percent of control values for each experiment.  
 The number of animals used appears in parenthesis.

Hours after infection		Exper. I (4)		Exper. II (3)		Exper. III (4)	
		24	48	24	48	24	48
Lactase	Jejunum	6	2	88	24	24	45
	Ileum	24	26	59	18	100	67
Maltase	Jejunum	23	28	92	89	100	100
	Ileum	74	22	70	23	100	100
Invertase	Jejunum	13	17	14	3	100	57
	Ileum	97	19	25	3	100	20
Trehalase	Jejunum	17	16	64	5	80	39
	Ileum	62	47	7	5	77	17

# LACTOSE TOLERANCE TEST





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